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THE METHOD OF SHADOW-CASTING IN PHOTOMICROGRAPHY

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Plate VIII

ABSTRACT. A discussion of the shadow-casting method in increasing contrast of unstained biological objects for use in optical microscope is presented A calculated quantity of silver is deposited on the specimen at an angle in this technique and estimates are made for the thickness that can be profitably deposited to provide proper opacity and contrast Optical micrographs of the protozoa L Donovani when shadow-cast with different thickness of silver are reproduced.

INTRODUCTION

The observation of unstained bacteria or protozoa with the usual optical microscope requires the closing down of the substage illuminator to a very narrow aperture in case of bright field illumination with widely convergent light or recourse has to be taken to dark ground illumination. This narrowing down of condenser aperture decreases the obliquity of the illuminating beam and hence increases the contrast of the image against the background brightness. The extent to which the condenser iris has to be stopped down depends on the opacity and refractive properties of the object imaged· For micro-organisms, the iris diaphragm may have to be closed down to such an extent that diffraction fringes become prominent around the contours which tend to spoil the image quality. In any case, the gain in contrast achieved by this process of constricting the illumination is at the expense of resolution of the instrument together with a possible increase in visual discomfort arising out of a large decrease in brightness of the microscope field. The usual procedure to obvia'e this difficulty is by the use of stains which enhance absorption contrast of the image. However, staining methods do not give any idea of the surface structure of the organisms, for which purpose other methods have to be employed. One of these methods, which has found extensive use in electron microscopy, is the shadow-casting technique of Williams and Wyckoff (1946). This method is not restricted to electron microscopy alone but can be employed to yield useful information in the case of optical microscopy of surfaces (Scott and Wyckoff, 1949). The present paper indicates how surface characteristics of unstained protozoa can be obtained by the

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employment of shadow-casting technique for enhancing contrast as well as revealing surface details without imposing too much restriction on the aperture of illumination.

Briefly, the method of shadow-casting consists of depositing a thin film of metal on the specimen at an angle and then observing it in the microscope. The specimen is placed in an evacuated chamber and the metal is evaporated from a hot tungsten coil situated at a suitable distance from the specimen. With a sufficiently good vacuum, the metal atoms proceed in straight lines and condense on the specimen from an angle. A deposit of heavy metal thus builds up on the exposed side of the specimen and radiation shadows free from metal are produced in the background behind high details. Due to the obliquity of the beam, the condensed metal film is of varying thickness over the surface according to the heights of the surface irregularities and these become prominent in the micrograph which presents a three dimensional appearance together with an increase in the contrast of the image. Further, from the length of the shadows and the geometry of the experimental arrangement, the average height of the specimen and also differences in surface elevation can be estimated. In the present paper, the conditions for an optimum deposit of metal have been discussed with particular reference to the parasite Leishmania Donovani (flagellate state) and photo-micrographs of the organism shadow-cast with silver have been obtained.

THICKNESS OF SHADOWED FILM OF METAL AND CONSIDERATIONS OF CONTRAST

The technique of shadow-casting is essentially similar for optical as well as electron microscopy. The difference lies only in the nature and amount of metal used. The nature of metal selected for shadowing in electron microscopy is primarily determined by the absence of structure in the shadow-cast film and considerations of electron scattering power. Very thin films, with thickness of the order of 10 A. U. are usually required (Drummond, 1949) for electron microscopy. As light has greater penetrating power than electrons, the metal coating can be made thicker for optical microscopy and since the structure present in the shadowed-film is not of primary importance here, metals like silver, which are easily evaporated, can be used, The amount of silver that can be deposited without appreciable restriction of the available illumination can be estimated as follows.

The theory of passage of plane electro-magnetic wave through a homogeneons, isotropic absorbing medium shows that if I_0 is the intensity of the incident radiation, the intensity I after travelling a distance t through the medium is given by

$$I = I_0 e^{-4\pi kt/\lambda} \qquad \ldots \quad (1)$$

where λ is the wavelength of incident light in vacuum and k is the extinction coefficient determining absorption in the metal. For silver, $k = 3.638$

for the *D*-line of sodium ($\lambda 5893$ Å.) as determined by Minor (1903). Assuming this value of k, the variation of transparency of silver films, *i.e* the ratio I/I_0 with thickness t, has been calculated from equation (1) and plotted in Fig. 1. This curve is strictly applicable only for the *D*-line of sodium and

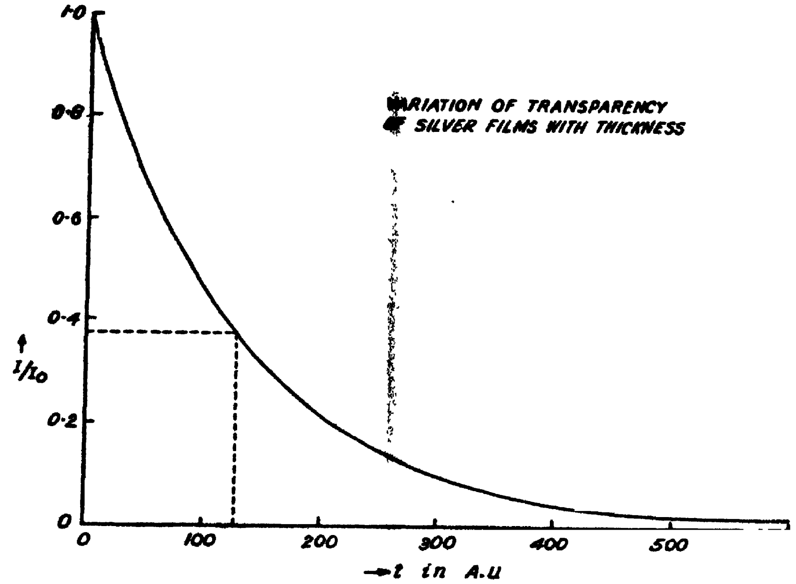


FIG. 1

for silver films, as k in equation (1) varies with wavelength and nature of the metal. However, the variation of k with λ for silver in the visible region is not very great and the conclusions that follow are applicable approximately to the whole of visible spectrum.

To deduce the maximum limit for the thickness of silver that can be deposited, we define a 'depth of penetration'. This is the thickness t_m which reduces light intensity by the factor $e^{-4\pi}$, that is, the amplitude is reduced by a factor $e^{-2\pi}$. From (1), we immediately get $t_m = \frac{\lambda}{k} \simeq 1600$ A.U. This is the thickness of silver that will reduce incident intensity by about 3.5×10^{-6} and will thus screen off all transmisson in the visible region.

Another definition which is useful is the 'penetration thickness' δ in which the light intensity drops to $\frac{1}{e}$ th of its inital value. From equation (1) we get

$$\delta = \lambda/4\pi k = 129 \text{ A.U.} \qquad \ldots \quad (2)$$

Let us now suppose that a specimen of height h and width b mounted on a slide is shadowed obliquely by evaporation of metal placed at M, the shadowing beam striking the slide at an angle α (Fig. 2) For consideration of metal deposit we may consider three regions : (*a*) specimen surface of area $h.b$

facing the beam. (b) regions of shadow cast by the specimen and (c) open portions of the slide exposed to the beam and away from all obstructions.

Let r cm be the distance of the source from the slide. r may be considered approximately constant over the slide, provided it is located at a sufficient distance away.

The solid angle subtended by the exposed side of the specimen at the source is then $h.\ b.\ \cos\alpha/r^2$ and since $M/4\pi$ is the mass of metal (in grams) evaporated per unit solid angle, the amount deposited on the specimen is $\frac{M}{4\pi}.\frac{h\ b \cos\alpha}{r^2}$. The thickness t_B in A. U. that is built up on the exposed side of the specimen is then given by

$$t_B = \frac{M \cos\alpha}{4\pi r^2 \rho} \times 10^8 \qquad \ldots \quad (3)$$

where ρ is density of metal in gms/cm^3.

It is usual to include a factor of 3/4 from considerations of efficiency of evaporation, so that in actual case (3) becomes

$$t_B = \frac{3}{4}.\frac{M \cos\alpha}{4\pi r^2 \rho} \times 10^8 \text{ A.U.} \qquad \ldots \quad (4)$$

For the region (c), which constitutes the background, we get from similar considerations the thickness t_B of metal as

$$t_B = \frac{3}{4}.\frac{M \sin\alpha}{4\pi r^2 \rho} \times 10^8 \text{ A.U.} \qquad \ldots \quad (5)$$

If the specimen is of length l, then the top of the specimen of area $l.\ b$ will be covered with the thickness t_B of metal.

The region (b) behind high details will be the shadow region completely free from metal. The length of shadow l' is then given by the relation.

$$l' = h \cot\alpha \qquad \ldots \quad (6)$$

and is measured perpendicularly to the surface in consideration.

Let us now make the following assumptions: (a) The specimen contains details which can be resolved by the optical microscope. Thus the minimum height of a surface irregularity can be taken to be 0.2μ, which is the resolving power of the microscope for visible radiation $\lambda 5550$ Å. and (b) the specimen has got no appreciable absorption for light rays ; which implies that the unshadowed object has got negligible contrast. Contrast of the object is only brought about by absorption in the shadowed silver film.

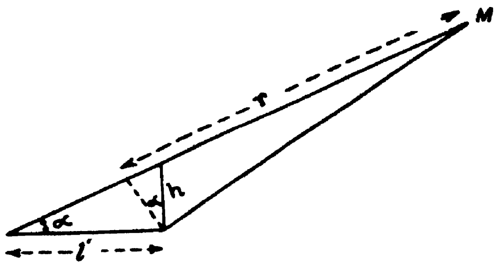
Now, the contrast g of an object against its surroundings is defined by the relation

$$g = \frac{B_0 - B}{B_0} \qquad \ldots \quad (7)$$

where B_0 is the brightness of the background and B is the brightness of the object. This holds for visual observation only. In case of photographic image, the B's are replaced by transmission of the different parts of the negative, the transparency ratio in a photographic, negative corresponding to a brightness ratio in the subject being given by the relation

$$\frac{T}{T_0}=\left(\frac{B_0}{B}\right)^{\gamma} \quad \ldots \quad (8)$$

where γ is obtained from the straight portion of the H and D characteristic of the negative material where the densities are assumed to lie. T and T_0 are respectively the transparencies of the light and dense portions of the negative. Expression (8) immediately shows that a detail invisible to the eye can show up in the negative, the gamma-value of a process panchromatic plate can be as great as 3.0. In microscopy, the field brightness is usually about 1000 candles/cm^2 At this level of illumination, the contrast sensitivity of the average human eye is approximately 10 per cent which means that two contiguous areas with brightness difference of 10 per cent can just be perceptible. Accordingly, we assume the minimum perceptible contrast to be 10 per cent and from Fig. 2 we find that if the film thickness



Shadowing at an oblique angle

FIG. 2

is reduced below about 13 A. U., the contrast between a shadow and the contiguous area will be less than 10 per cent and the shadows will be indistinguishable from the background. Assuming a usual gamma-value of unity, this then will be the minimum value of film thickness for visual as well as photographic records.

We thus see that enhancement of contrast of objects can be brought about by silver shadowing over wide limits. For example, for a thickness $t=130$ A. U. the contrast is found to be 63.2 per cent, which increases still further as t is increased.

Again, the contrast between a specimen detail of height h facing the beam and its immediately contiguous background is given by the relation

$$gs=\frac{I_B-I_S}{I_B}=1-e^{-h/\delta} \quad \ldots \quad (9)$$

where I_B is the intensity transmitted by the background which has a thickness t_B and I_S is the intensity transmitted through a thin film of metal of height h deposited at a thickness t, on the specimen, given by $I_S = I_B e^{-(h+t)\sin\alpha/\delta}$,

where $A = \frac{3}{4} \cdot \frac{M}{4\pi r^2 \rho}$ Since δ for silver is only 130 A. U., and the specimen is assumed to be not less than $0.2\,\mu$ or 2000 A. U. high, we get $g_s = 1 - e^{-15}$, which shows that the object has nearly cent per cent contrast against the background. Thus there appears to be no difficulty arising from image contrast in silver shadowed objects and the substage iris can always be adjusted to the optimum value for critical microscopy. Since silver shadowing and the resulting deposit of silver on the side of the object facing the beam will produce a modification of dimension on one side, which for a given film thickness t is $t_s = A \cos\alpha$, the film thickness has to be adjusted to as small a value as is compatible with adequate shadow contrast. The contrast of the image against its shadow is given by $g_t = 1 - e^{-A \sin\alpha/\delta}$ and as we found earlier, for $g_t = 0.1$, $A \sin\alpha$ should not be less than 13 A. U. The contrast increases as $A \sin\alpha$, which can be seen from the attached micrographs of L. Donovain (Plate VIII). A limit to the increase in $A \sin\alpha$ can be put by adopting a minimum for the thickness of the shadowing layer t_S. If we define this thickness to be of such magnitude that it must not alter the actual dimension by more than 10 per cent, we at once find that a $0.2\,\mu$ specimen is not to have a coating more than 200 A. U. thick on its exposed side. Changing α from $\tan^{-1}\frac{1}{2}$ to $\tan^{-1}\frac{1}{5}$, we find that the background depth t_B ranges from 100 A. U. to

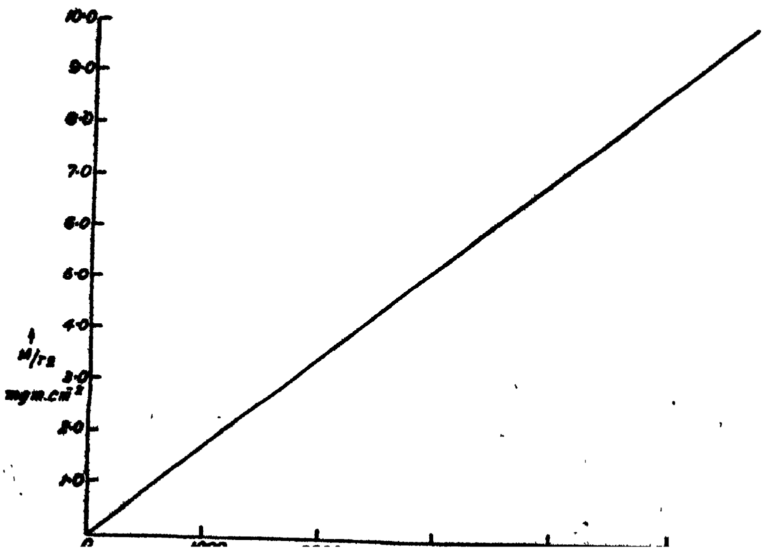


FIG. 3

40 A. U. respectively and the shadow contrast then ranges from 55 to a little more than 25 per cent. Shadowing considerations for deciding

(*a*) (*b*) (*c*)

(*d*) (*e*) (*f*)

All the photomicrographs of L. Donovani were taken with Leitz Panphot camera microscope using their $\frac{1}{12}''$ objective with numerical aperture 1 30. The magnification of all figures is 1830 X. All the specimens except that of Fig. *a* were fixed in osmic acid and subsequently washed with water prior to shadow-casting.

Fig. *a*. L. D. flagellate stained with Leishman's stain ; unshadowed.

Fig. *b*. L. D. flagellate shadow-cast with silver. Computed thickness 50 A. U. at an angle of $\tan^{-1}\frac{1}{3}$.

Fig. c. Do. Computed thickness 112 A.U. at $\tan^{-1}\frac{1}{5}$

Fig. *d*. Do. Computed thickness 200 A.U. at $\tan^{-1}\frac{1}{4}$.

Fig. *e*. Do. Computed thickness 250 A.U. at $\tan^{-1}\frac{1}{5}$.

Fig. *f*. Do. Computed thickness 330 A.U. at $\tan^{-1}\frac{1}{3}$.

$A \sin \alpha$ for optical microscopy should be made within these limits. For angles α not greater than $15°$, $\sin \alpha$ can be replaced by $\tan \alpha$ and a curve like Fig. 3 plotted from equation (5) will be helpful in obtaining any of the four quantities when the other three are fixed.

The above discussion indicates a thickness of 50-100 A.U. of silver at angles α from $\tan^{-1} \frac{1}{4}$ to $\tan^{-1} \frac{1}{2}$ as useful for routine shadowing of specimens which are suspected to possess surface irregularities. For the visualisation of low details, long shadows are useful but since any given specimen for optical microscopy will, in general, contain details with variable heights, the possibility of overlapping of these shadows must be borne in mind when shadowing at very oblique angles.

For quantitative work, the source should subtend as small a solid angle at the specimen as possible so that the condition of a point source assumed in the calculation of thickness is approximated. This implies that the source of metal has to be made as small as possible and the distance of the spcimen from the source as large as possible. Further, devices for measuring the angle α with reasonable accuracy have to be provided.

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